

# The mammalian CHORD-containing protein melusin is a stress response protein interacting with Hsp90 and Sgt1

Mauro Sbroggi<sup>a</sup>, Roberta Ferretti<sup>a</sup>, Elena Percivalle<sup>a</sup>, Malgorzata Gutkowska<sup>b,c</sup>, Alicja Zylcz<sup>b</sup>, Wojciech Michowski<sup>b,c</sup>, Jacek Kuznicki<sup>b,c</sup>, Federica Accornero<sup>a</sup>, Beniamina Pacchioni<sup>d</sup>, Gerolamo Lanfranchi<sup>d</sup>, Jorg Hamm<sup>a</sup>, Emilia Turco<sup>a</sup>, Lorenzo Silengo<sup>a</sup>, Guido Tarone<sup>a</sup>, Mara Brancaccio<sup>a,\*</sup>

<sup>a</sup> Department of Genetics, Biology and Biochemistry, University of Torino, Molecular Biotechnology Center, via Nizza, 52, Torino, Italy

<sup>b</sup> International Institute of Molecular and Cell Biology in Warsaw, 4 Trojdena Street, Warsaw, Poland

<sup>c</sup> Nencki Institute of Experimental Biology, Warsaw, Poland

<sup>d</sup> CRIBI Biotechnology Centre and Biology Department, University of Padova, Padova, Italy

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**Abstract** Melusin is a mammalian muscle specific CHORD containing protein capable of activating signal transduction pathways leading to cardiomyocytes hypertrophy in response to mechanical stress. To define melusin function we searched for molecular partners possibly involved in melusin dependent signal transduction. Here we show that melusin and heat shock proteins are co-regulated. Moreover, melusin directly binds to Hsp90, a ubiquitous chaperone involved in regulating several signaling pathways. In addition, melusin interacts with Sgt1, an Hsp90 binding molecule. Melusin does not behave as an Hsp90 substrate but rather as a chaperone capable to protect citrate synthase from heat induced aggregation. These results describe melusin as a new component of the Hsp90 chaperone machinery.

*Structured summary:*

MINT-6538515:

*melusin* (Q9R000) physically interacts (MI:0218) with *Hsp90* (P07901) by anti bait co-immunoprecipitation (MI:0006)

MINT-6538566, MINT-6538556:

*Hsp90* (P07901) physically interacts (MI:0218) with *melusin* (Q9R000) by cross-linking studies (MI:0030)

MINT-6538524:

*melusin* (Q9R000) physically interacts (MI:0218) with *Sgt1* (Q9CS74) by anti tag co-immunoprecipitation (MI:0007)

MINT-6538595:

*Hsp90* (P07901) physically interacts (MI:0218) with *melusin* (Q9R000) by enzyme linked immunosorbent assay (MI:0411)

MINT-6538543:

*melusin* (Q9R000) physically interacts (MI:0218) with *Sgt1* (Q9CS74) by anti bait co-immunoprecipitation (MI:0006)

MINT-6538580:

*melusin* (Q9R000) physically interacts (MI:0218) with *Hsp90* (P07901) by surface plasmon resonance (MI:0107)

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**Keywords:** Melusin; CHORD containing proteins; Hsp90; Sgt1

## 1. Introduction

Melusin is a muscle specific protein originally identified for its ability to bind to the  $\beta 1$  integrin cytoplasmic domain [1]. Melusin is characterized by two N-terminal CHORD domains and by a C-terminal CS domain, which is shared by metazoan CHORD containing proteins and SGT1 and also present in the co-chaperone p23 [2].

CHORD containing protein, rar1, has also been described in plants as a crucial component of the pathway mediating disease resistance [2]. Rar1 consists of two CHORD domains, but lacks the CS domain typically present in metazoan CHORD proteins.

Using loss and gain of function genetically modified mouse models we demonstrated that melusin is required to trigger cardiomyocyte hypertrophy in response to stress stimuli such as mechanical overload. Lack of melusin leads to reduced left ventricle hypertrophy and accelerates the evolution toward heart dilation in response to pressure overload [3]. On the other hand forced melusin expression in heart allows the development of sustained concentric hypertrophy and prevents the evolution toward heart failure [4].

In cardiomyocytes, melusin controls the phosphorylation state of the kinases ERKs, AKT and GSK3 $\beta$  and these pathways are required for activation of the hypertrophy response [4,5]. Since melusin does not possess a kinase or phosphatase domain, we search for melusin functional partners able to mediate melusin signal transduction. Using gene expression profiling and co-immunoprecipitation analysis, we demonstrated that melusin is co-regulated with heat shock proteins, binds to Hsp90 and Sgt1 and behaves like a chaperone.

## 2. Materials and methods

### 2.1. Cell culture and reagents

COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) (Invitrogen). C2C12 mouse skeletal muscle cell line was cultured in DMEM with 10% fetal calf serum (Invitrogen). Cells were induced to differentiate into myotubes by switching to culture medium with 2% horse serum (Invitrogen) for 6 days.

Hsp90 inhibition experiments were performed treating C2C12 myotubes with three different concentrations (0.5–1–2  $\mu$ g/ml) of radicicol (Sigma) for 24 h.

\*Corresponding author. Fax: +39 116706432.

E-mail address: [mara.brancaccio@unito.it](mailto:mara.brancaccio@unito.it) (M. Brancaccio).

COS cells were transiently transfected with expression vectors using DEAE/chloroquine transfection method and cells were lysed after 48 h.

Monoclonal antibody against melusin (clone 5E1) was produced in our laboratory by immunizing mice with recombinant GST–human melusin and its reactivity was characterized in western blot and immunoprecipitation. The epitope was mapped in the N-terminal CHORD I–II region. The following antibodies were used: Hsp90 (# SPA-835), Hsp70 (# SPA-810), Hsc70 (# SPA-815), Hsp40 (# SPA-400) (Stressgen), AKT (# 9272), MEK1/2 (# 9122) (Cell Signaling Technology), Src and GST (Santa Cruz Biotechnology), GAPDH (# MAB374 Chemicon), Sgt1 (# 612104 BD Biosciences), Flag M2 (# F1804) and Myc clone 9E10 (Sigma), vinculin (Sigma).

## 2.2. Expression vectors and recombinant protein production

To produce recombinant melusin fragments fused to maltose binding protein (MBP), the nucleotide sequence encoding for the amino acid residues 1–219 (CHORD I–II) and 211–320 (CS) were cloned in pMAL C2 vector. Expression vector encoding glutathione *S*-transferase (GST) fused to melusin was prepared as previously described [1]. Vectors encoding GST–Hsp90 $\alpha$  fragments [N (9–236), M (272–617), or C domains (629–732)] [6] were kindly provided by prof. W.C. Sessa (Yale University). Flag-tagged Sgt1 cDNA was kindly provided by Marcin Nowotny (International Institute of Molecular and Cell Biology, Warsaw).

MBP and GST fusion proteins were produced in *Escherichia coli* BL21 bacterial strain and then purified on a Sepharose amylose (New England Biolabs) or Sepharose glutathione column (GE Healthcare) according to the manufacturer's instructions. MBP–Hsp90 $\alpha$  was produced as a recombinant protein and purified as described [7]. After purification, the MBP tag was cleaved out with Xa protease.

## 2.3. Stress stimuli in vivo

Wild type anesthetized mice were subjected to heat shock by immersion in a water bath at 41.5 °C for 30 min [8]. Mice were allowed to recover for 10–30 h, killed and hearts and skeletal muscles were collected and analyzed by western blot.

Mechanical stimuli were obtained by subjecting hearts to pressure overload induced by surgical constriction of the transversal aorta as previously described [5].

## 2.4. Co-immunoprecipitation and western blot analysis

Co-immunoprecipitation experiments were performed both on differentiated C2C12 myogenic cell line in culture and on heart tissue extracts.

C2C12 differentiated myotubes were washed twice with PBS and lysed in 20 mM HEPES pH 7.4, 0.1% NP-40, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EDTA. Lysates were subjected to three freeze and thaw cycles. After centrifugation at 14000 rpm at 4 °C for 10 min, the supernatants containing 3 mg of total protein extract were immunoprecipitated overnight at 4 °C with 5  $\mu$ g of the selected antibody, followed by incubation with Protein G Sepharose.

Cross-linking experiments were performed by incubating C2C12 cells for 10 min at room temperature in PBS containing 200  $\mu$ g/ml of the membrane permeable cross-linker Dithio-bis succinimidylpropionate (DSP) (Pierce). Cross-linked cells were washed twice in PBS and lysed in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). DSP untreated C2C12 were lysed in the same buffer and were used as control. After centrifugation at 14000 rpm at 4 °C for 10 min, the supernatants containing 4 mg of total protein extract were incubated with 5  $\mu$ g of the selected antibody for overnight immunoprecipitation followed by incubation with Protein G Sepharose.

Mice were killed and hearts were rapidly collected, frozen and pulverized in liquid nitrogen in a mortar. Tissue powder was dissolved in 50 mM HEPES, 100 mM NaCl, 0.1% Deoxycholate, 0.1% Triton X-100, 2 mM EDTA, 1 mM EGTA. After centrifugation at 14000 rpm at 4 °C for 10 min, the supernatants containing 4 mg of total protein extract were incubated with 5  $\mu$ g of the selected antibody for immunoprecipitation followed by incubation with Protein G Sepharose.

To investigate melusin–Sgt1 interaction we used COS cells transfected with Myc–melusin and flag–Sgt1. Transfected COS cells were washed twice with PBS and lysed in TBS (150 mM NaCl, 50 mM

Tris–HCl, pH 7.4), 0.5% Triton X-100. Two milligrams of total proteins were incubated overnight at 4 °C with 5  $\mu$ g of the appropriate antibody.

All the lysis buffers used were added of 10  $\mu$ g/ml leupeptin, 4  $\mu$ g/ml pepstatin, 0.1 TIU/ml aprotinin, 1 mM PMSF, 1 mM NaVO<sub>4</sub> and 10 mM NaF.

For total protein extracts analysis, C2C12 cells and pulverized mouse hearts were lysed in TBS, 1% Triton X-100.

Immunoprecipitated samples of total protein extracts were analyzed by SDS–PAGE and western blot developed by chemiluminescent reagent, LiteAblo (Euroclone).

Quantification of band intensities was done by densitometric analysis with Quantity One software (Bio-Rad).

## 2.5. Surface plasmon resonance analysis

Surface plasmon resonance (SPR) experiments were performed with a Biacore 2000 system (Biacore, Inc.). Recombinant melusin fragments (MBP–CHORD I–II, MBP–CS and MBP alone) were employed as ligands immobilized by amine group coupling on a CM5 biosensor chip (Biacore, Inc.) as indicated by manufacturer instructions. GST–fusion HSP90 $\alpha$  fragments (C, M and N domains) [6] were used as soluble analytes. All analytes were dissolved in running buffer and binding experiments were performed at 25 °C in running buffer with a flow rate of 20  $\mu$ l/min. The amount of immobilized MBP–CS, MBP, MBP–CHORD I–II were 2532, 2253, and 2806 resonance units (RU) respectively. The binding to MBP–CHORD I–II results in a response of  $\sim$ 100 RU with 7  $\mu$ M N-terminal Hsp90. All the binding curves were corrected for the signal of a cell without proteins coupled on the matrix. The binding experiment performed with the Biacore was of qualitative nature. We have not attempted so far to determine quantitatively the kinetic parameters of the interaction.

## 2.6. ELISA binding assay

The assay was performed as described [9]. Briefly, 0.5  $\mu$ g/well of Hsp90 $\alpha$  were added to a 96-well microtiter plate in 50  $\mu$ l of Coating Buffer (CB: 25 mM HEPES pH 7.2, 100 mM KCl). After 1 h incubation and washing the indicated proteins (GST–melusin or GST) were added in 50  $\mu$ l of Reaction Buffer (RB: 25 mM HEPES pH 7.2, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 5% glycerol, 2 mM DTT, 0.01% Triton X-100, 1 mg/ml of BSA) and the reaction took place for 1 h at room temperature. Where indicated the RB was also supplemented with 4 mM ATP. After washing the appropriate antibodies (melusin monoclonal or GST rabbit polyclonal antibodies) were added for detection of the interactions. Each experiment was repeated three times and the standard deviation was calculated as less than 5%.

## 2.7. Citrate synthase aggregation–protection assay

75 nM citric synthase (Sigma) in 40 mM HEPES buffer pH 7.5 was incubated at 43 °C for 30 min. Incubation was performed in Shimadzu RF 5301 spectrofluorimeter in 0.5 ml quartz cuvette (Helma). Light scattering was measured every 20 s, slit opening 3/3 mm, excitation and emission wavelength set to 465 nm. GST–melusin and control proteins were added at the indicated concentrations during incubation [10]. Control experiment showed no contribution to the light scattering of GST and GST–melusin upon heating (see Fig. 5b).

## 2.8. Transcriptome analysis

Gene expression profile was performed on a mouse microarray platform with a collection of 13443 70mer oligonucleotides (Qiagen-Operon, version 1.1). Preparation of the microarray, RNA labeling, hybridization and detection of differentially expressed transcripts were performed as described [4].

## 2.9. Real-time PCR

Total RNA was extracted from mouse hearts using TRIZOL reagent (Invitrogen). 1  $\mu$ g of total RNA was retrotranscribed to cDNA with Random Primers (New England Biolabs) using the M-MLV Reverse Transcriptase (Invitrogen) according to manufacturer instructions. Multiplex Real-Time PCR was performed on AB 7300 Real-Time PCR system using Applied Biosystems TaqMan Gene Expression Assays for the targets Hsp70 (Assay ID: Mm00434069\_s1), Hsp90 $\alpha$  (Assay ID: Mm00833431\_g1) and Hsp90 $\beta$  (Assay ID: Mm00658568\_gH). Eukaryotic 18S rRNA (Applied

Biosystems, 4319413E) was used as endogenous control to normalize targets expression level. Relative quantitation of gene expression level was analyzed with SDS RQ Study software (Applied Biosystems).

### 3. Results

#### 3.1. Melusin is co-regulated with heat shock proteins

Co-regulated genes are often functionally related [11]. Thus, to gain insight into melusin signal transduction mechanisms, we performed microarray analyses comparing the gene expression profiles of hearts from mice lacking [3] or overexpressing melusin [4] with the related wild type. The number of genes upregulated or downregulated was low in both mouse strains (data not shown), demonstrating that the absence or the overexpression of melusin slightly modifies the heart expression profile in basal condition. This is in line with our previous results, demonstrating that lack of melusin or its overexpression does not significantly alter tissue architecture and basal heart function.

When the gene fingerprints of the two mouse strains were compared to each other, only one gene, coding for heat shock protein 70, was found to be upregulated in melusin overexpressing and downregulated in melusin null hearts.

To further investigate this aspect we performed quantitative RT-PCR analysis on Hsp70 and its functionally correlated

molecules Hsp90 $\alpha$  and Hsp90 $\beta$ . As shown in Fig. 1a, Hsp70 mRNA levels were 2-fold decreased in melusin null and 2,3-fold increased in melusin overexpressing hearts compared to wild type. Moreover Hsp90 $\alpha$  mRNA levels were also significantly decreased in melusin null and increased in melusin overexpressing hearts. On the other hand Hsp90 $\beta$  mRNA levels did not correlate with melusin expression.

To verify if the co-regulation between melusin and Hsp70 and Hsp90 occurred also at the protein level, we performed western blot analysis, showing that Hsp70 was downregulated (25% decreased) in melusin null hearts, while was upregulated (2-fold) in melusin overexpressing hearts compared to wild type controls (Fig. 1b). Similar results were obtained for Hsp90 (Fig. 1b).

Heat shock protein expression is enhanced by multiple stress stimuli, including heat shock and mechanical load. We thus investigated whether melusin expression was up-regulated by these stimuli by subjecting mice to either *in vivo* heat shock or to mechanical load.

The results showed that while Hsp70 and Hsp90 were strongly up-regulated in heart 10 and 30 h after heat shock, melusin expression was not significantly modified (Fig. 2a). Similar results were obtained analyzing skeletal muscles (tibialis anterior, gastrocnemius and quadriceps) at the same time points (data not shown).

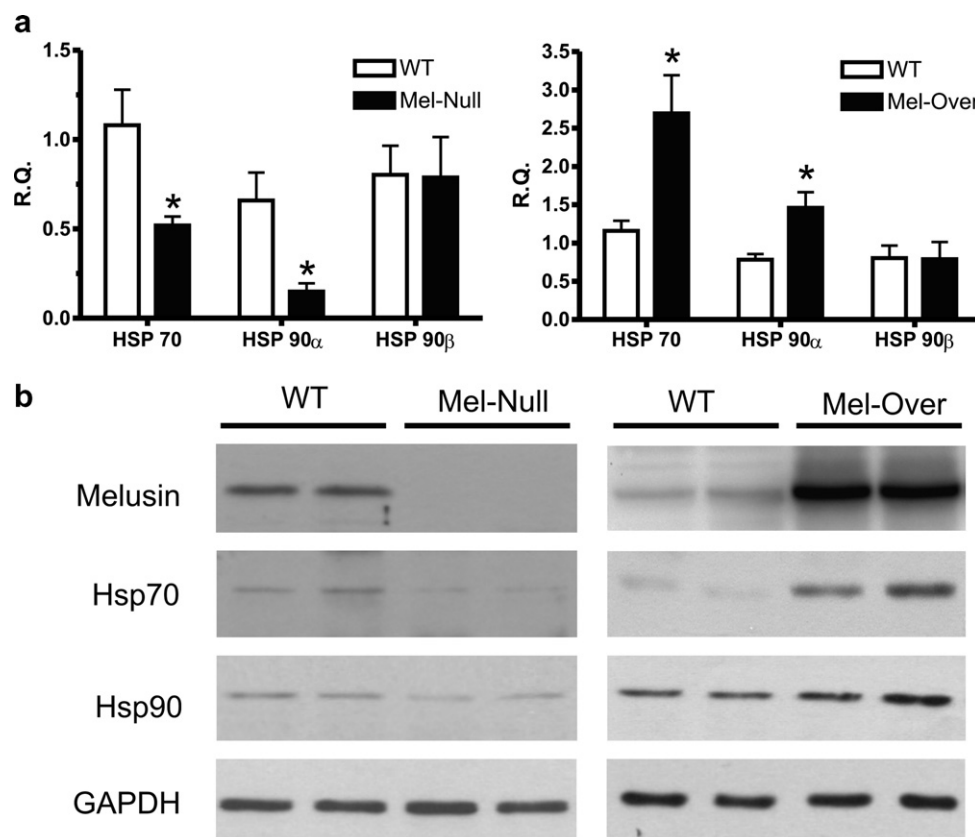


Fig. 1. Hsp70 and Hsp90 are co-regulated with melusin *in vivo*. (a) Hearts from melusin null [3] or melusin overexpressing mice [4] were examined by real time PCR for Hsp70 and Hsp90 $\alpha$  and  $\beta$  expression. Bars show mRNA relative quantitation (R.Q.). Four mice for each group were examined (\* $p < 0.05$ ). (b) Hearts from melusin null or melusin overexpressing mice were examined by western blot for Hsp70 and Hsp90 expression. Melusin and GAPDH expression were evaluated as controls. Eight mice for each group were examined; in the figure representative results on two mice per group were shown.

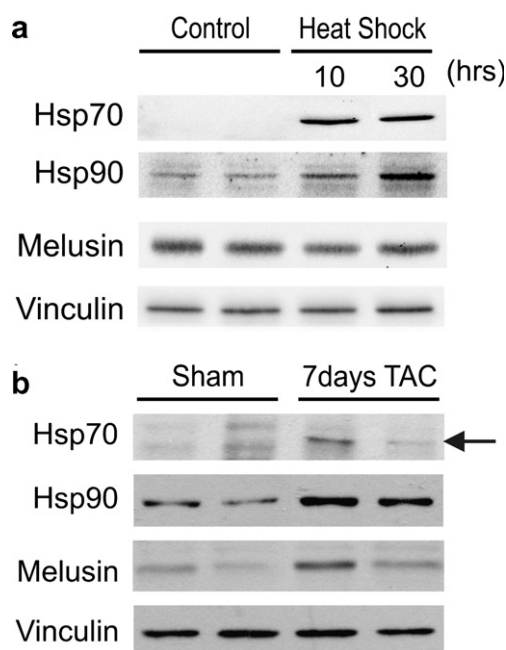


Fig. 2. Melusin is co-regulated with heat shock proteins only in response to mechanical stimuli. (a) Wild type anesthetized mice were subjected to heat shock by immersion in a water bath at 41.5 °C for 30 min [8]. Mice were allowed to recover for 10 and 30 h and then hearts were collected and analyzed by western blot. (b) To impose mechanical overload to cardiac muscle, mice were subjected to transversal aortic constriction (TAC) for 7 days. Sham operated animals were used as control. Hsp70, Hsp90 and melusin levels were revealed by western blot analysis. Vinculin was used as internal sample loading control. Four mice for each group were examined; in the figure representative results on two mice per group were shown.

On the other hand when mechanical load was imposed to the left ventricle walls, both heat shock proteins like Hsp70 and Hsp90 and melusin were significantly up-regulated (Fig. 2b).

Thus, melusin behaves like a stress induced protein in response to mechanical stretch but not to heat shock treatment.

### 3.2. Melusin binds to Hsp90

Proteins whose expression is co-regulated are functionally related and often capable of reciprocal interaction. Thus, we tested the ability of melusin to bind to Hsp90 and Hsp70 using the myogenic cell line C2C12. After serum starvation, C2C12 myoblasts fuse into myotubes and express melusin [1]. As shown in Fig. 3a when melusin was immunoprecipitated with a specific antibody from differentiated C2C12 cell extracts, Hsp90 was found in the immunoprecipitate. Conversely Hsp70 was not detectable in the immunocomplex (Fig. 3a). To avoid the possibility that Hsp90–melusin interaction was caused by partial melusin denaturation during protein extraction, we performed cross-linking experiment in intact cells with the membrane-permeable cross-linker Dithio-bis succinimidylpropionate (DSP). Differentiated C2C12 were treated with DSP for 10 min, lysed in RIPA buffer (see Section 2) and immunoprecipitated with an anti-Hsp90 antibody. Western blot analysis on the reduced samples demonstrated the presence of melusin in the immunocomplex (Fig. 3b). Untreated cells lysed in RIPA buffer did not show melusin co-immunoprecipitation, since the non-

cross-linked complex is not preserved in high detergent conditions. Moreover, to verify if this interaction take place in vivo, we performed immunoprecipitation of Hsp90 from mouse heart extracts. As shown in Fig. 3c, western blot analysis on the immunoprecipitated samples clearly demonstrated melusin–Hsp90 association.

To test whether the melusin–Hsp90 interaction is direct and to map the molecular regions involved, we used surface plasmon resonance (SPR). Both N-terminal (CHORD I–II) and C-terminal (CS) melusin fragments were immobilized on the Biacore sensor chip and either N-terminal (N), middle (M) or C-terminal (C) Hsp90α domains [6], were used in the liquid phase. The N-terminal Hsp90 domain was found to bind to CHORD I–II of melusin (Fig. 3d) in a dose dependent manner (Fig. 3e), while no binding was detected for M and C Hsp90 domains (Fig. 3d). None of the three Hsp90 fragments interacted with melusin CS domain (Fig. 3f and data not shown), demonstrating that melusin CHORD I–II domain is sufficient for the direct binding to N domain of Hsp90.

To further demonstrate the direct association between melusin and Hsp90 we performed an enzyme linked immunosorbent assay (ELISA) with recombinant purified proteins as previously described [9]. The results clearly demonstrate that full length melusin is able to bind Hsp90 (Fig. 3g) and that the interaction occurs via Hsp90 N domain (Fig. 3h). Interestingly melusin binding to the entire Hsp90, but not to its N domain, is strongly enhanced by the presence of ATP (Fig. 3g and h). This effect was inhibited by the preincubation of Hsp90 with radicicol, which competes for ATP binding to Hsp90 (data not shown).

### 3.3. Melusin is not an Hsp90 client protein

Hsp90 binding proteins belong to two different categories: Hsp90 substrates or Hsp90 co-chaperones, i.e. factors that assist or modify Hsp90 activity [12].

Hsp90 is able to bind and protect from degradation a wide range of substrates or “client proteins”, most of which are involved in signal transduction. Treatment with radicicol, an inhibitor of the Hsp90 ATPase activity, causes the release and degradation of client proteins. In contrast Hsp90 co-chaperones are not degraded by radicicol treatment, but they are rather induced by an indirect mechanism of gene transcription enhancement [13]. After 24 h treating of C2C12 differentiated myotubes with radicicol, the level of well known Hsp90 client proteins, such as Akt, Src and MEK [14], are drastically decreased while chaperones (Hsp90 and Hsp70) and co-chaperones (Hsp40) are up-regulated (Fig. 4). Interestingly, melusin protein levels were not decreased by radicicol treatment, but were enhanced at the higher radicicol dose used in the experiment (Fig. 4). These results indicate that melusin is not a client protein. Moreover, radicicol induced upregulation suggests its possible role as a chaperone or as a co-chaperone.

### 3.4. Melusin behaves as a chaperone

To test the possible chaperone behavior of melusin, we measured its ability to suppress aggregation of the thermosensitive enzyme citrate synthase. The presence of recombinant GST–melusin suppressed the heat-induced (45 °C) aggregation of citrate synthase in a dose dependent manner, while GST alone did not (Fig. 5a). This result demonstrates a melusin intrinsic chaperone activity.



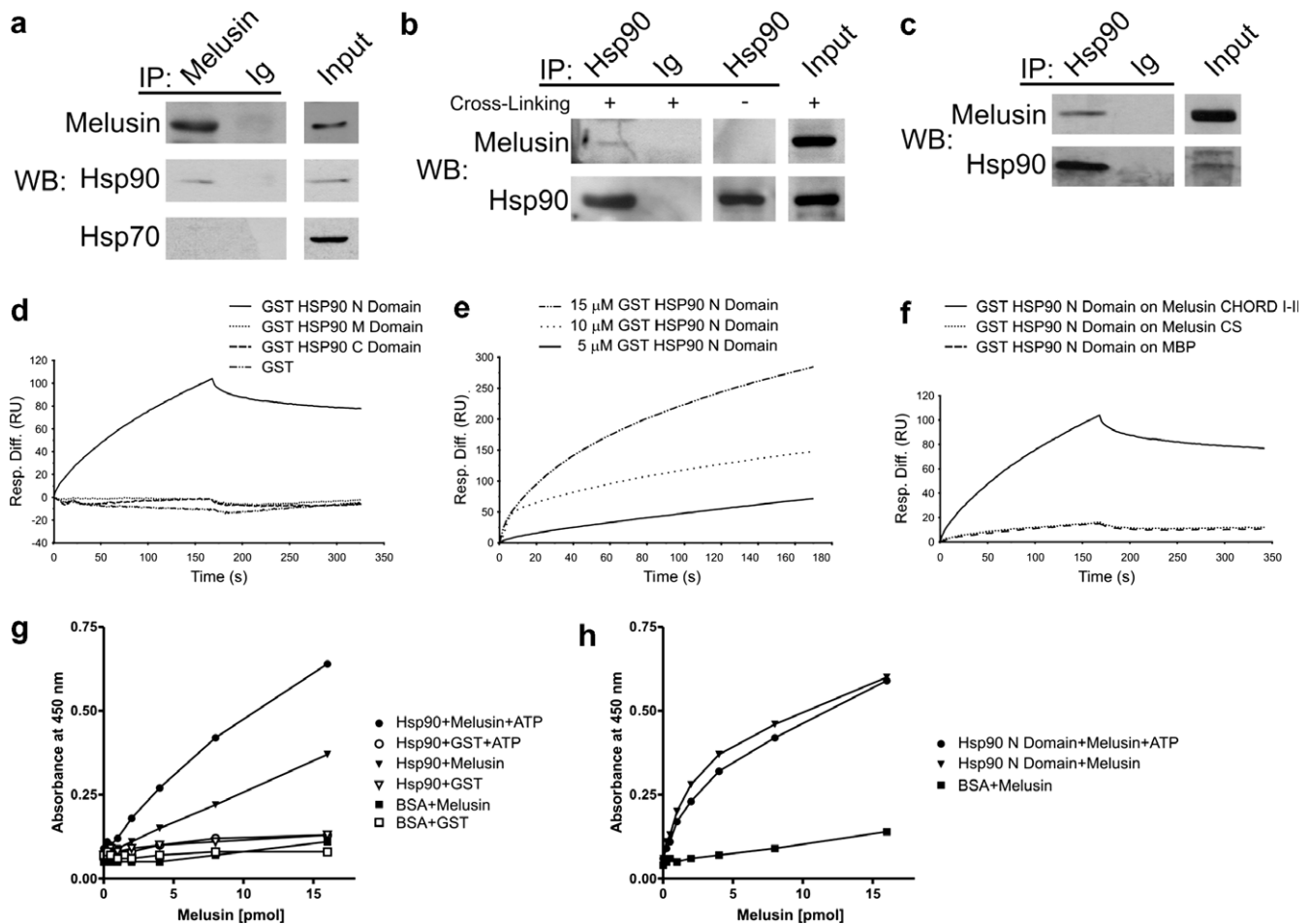


Fig. 3. Melusin and Hsp90 are binding partners. (a) Differentiated C2C12 cells were extracted in low detergent hypotonic buffer (see Section 2) and immunoprecipitated (IP) using a melusin specific antibody and control immunoglobulins (Ig). After separation by SDS-PAGE and western blotting (WB), proteins were detected with anti-melusin, anti-Hsp90 and anti-Hsp70 antibodies as indicated on the left. (INPUT: total cell extract). (b) C2C12 differentiated myotubes in culture were subjected to cross-linking by the membrane permeable and thio-cleavable crosslinker DSP. Cells were extracted with RIPA buffer (see Section 2) and soluble proteins were immunoprecipitated with an antibody against Hsp90 and with control immunoglobulins (Ig) from cross-linked cells. (c) Mouse heart protein extracts were immunoprecipitated using an anti-Hsp90 antibody and control immunoglobulins (Ig). Samples were subjected to western blot analysis using anti-Hsp90 and anti-melusin antibodies, as indicated on the left. (INPUT: total cell extract). (d) The graph shows the results obtained with surface plasmon resonance (SPR) analysis when GST-Hsp90 $\alpha$  N domain, GST-Hsp90 $\alpha$  M domain, GST-Hsp90 $\alpha$  C domain and GST alone were used in liquid phase and tested for their ability to bind MBP-melusin CHORD I-II immobilized on the chip surface. (e) SPR binding curves obtained when GST-Hsp90 $\alpha$  N domain was added at three different concentrations on the MBP-melusin CHORD I-II coated chip, are shown. (f) The ability of GST-Hsp90 $\alpha$  N domain in the liquid phase to bind MBP-melusin CHORD I-II, MBP-melusin CS and MBP alone immobilized on the surface of the sensor chip, was tested with SPR analysis. (g) ELISA wells were coated with Hsp90 $\alpha$  recombinant protein or BSA. The indicated amounts ( $x$ -axis scale) of GST-melusin or GST alone were added in absence or presence of 4 mM ATP. For the detection of the complex formation an anti-GST antibody was used. (h) ELISA wells were coated with Hsp90 $\alpha$  N domain or BSA and incubated with the indicated amount of GST-melusin ( $x$ -axis scale) in absence or presence of 4 mM ATP. Detection of the complex formation was done by the use of an anti-melusin antibody.

### 3.5. Melusin interacts with Sgt1

CHORD domains of plant melusin homolog rar1 have been shown to bind Sgt1 [15], a well known Hsp90 interactor. Thus we investigated the possible interaction between melusin and Sgt1. To this purpose COS cells were transfected with plasmids coding for Myc tagged melusin. Immunoprecipitation of melusin with an anti-melusin antibody, followed by western blot analysis with an anti-Sgt1 antibody, revealed the presence of Sgt1 in the immunocomplex (see Fig. 6a). To perform reverse co-immunoprecipitation, we co-transfected COS cells with Myc tagged melusin and flag tagged Sgt1. Using an anti-flag antibody to precipitate Sgt1, we demonstrate melusin co-precipitation (Fig. 6b).

### 4. Discussion

CHORD containing proteins are a family of molecules originally identified in plant and mammals and are highly conserved during phylogenesis. In plants one gene coding for CHORD containing protein has been identified as a key component of the signalling pathway mediating innate immunity to pathogens [2]. In vertebrates a gene duplication event gave rise to two distinct CHORD containing proteins, melusin and chp-1 [3]. Chp-1 is a ubiquitous protein that has been shown to associate with Nod-1, a member of the Nod-like intracellular receptors involved in innate immunity in mammals [16], suggesting a conserved function for these proteins

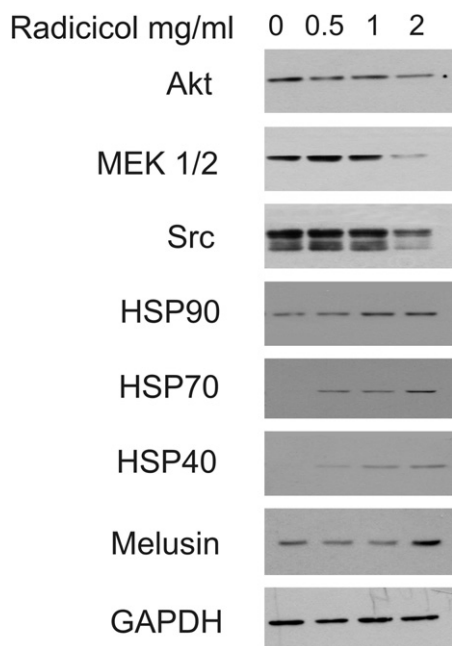


Fig. 4. Melusin is not an Hsp90 client protein. Differentiated C2C12 cells either untreated (0) or treated with different concentrations of radicicol (0.5; 1; 2 µg/ml) for 24 h, were analyzed by western blot for the expression of proteins indicated on the left. GAPDH was used as internal sample loading control.

during evolution. Melusin, on the other hand, is selectively expressed in striated muscle tissues and is involved in protecting heart from dilation and failure [5] by activating phosphorylation of signalling kinases such as AKT, GSK3 $\beta$  and ERK1/2 [5,4].

Here we show that melusin is co-regulated with heat shock proteins such as Hsp70 and Hsp90 and can form a complex with the latter one. Interestingly, we notice that, while the expression of Hsp90 and Hsp70 is regulated by different stress stimuli, melusin expression is regulated only by mechanical stretch but not by heat shock. This finding is in agreement with previous data showing that melusin is required in triggering

cardiomyocyte hypertrophy in response to mechanical load, but not to neurohumoral stimuli [5].

Hsp90 is a highly conserved heat shock protein, expressed in all eukaryotic cells. In addition to be up-regulated by stress stimuli, Hsp90 is one of the most abundant proteins in unstressed cells, where it is involved in protein trafficking and turnover as well as in signal transduction regulation. In the last years has become evident that the Hsp90 machinery is an essential multichaperone complex that regulates the degradation and the activation of over 100 substrates involved in signal transduction and transcriptional regulation [12]. Moreover, a key role is emerging for co-chaperone proteins in directing Hsp90 action on particular clients and consequently on specific signaling pathways [17,18].

By co-immunoprecipitation and cross-linking experiments, we demonstrated that melusin forms a complex with Hsp90. Moreover, using ELISA binding assay, we showed that such binding is direct and is strongly increased by ATP. In addition by surface plasmon resonance analysis we demonstrated that melusin CHORD I–II domain is sufficient to bind to Hsp90 amino terminal ATPase domain.

Conversely, Hsp70, another Hsp90 machinery component co-regulated with melusin during mechanical stress, was not detected in the complex. A possible explanation is that the interaction between Hsp70 and Hsp90 is transient and mediated by specialized co-chaperones [14].

Hsp90 binding partners belong to two different protein families: Hsp90 substrates, also called client proteins, and co-chaperones, which help Hsp90 in its action on substrates or mediate Hsp90-client recognition. Using Hsp90 inhibitors we show that melusin is not an Hsp90 client protein but rather behaves like a chaperone. In fact, melusin efficiently inhibits the heat induced aggregation of citrate synthase, clearly demonstrating that melusin possesses a chaperone activity per se. Interestingly, melusin contains in the C-terminal portion a CS domain, a protein motif present in p23 and Sgt1, two known Hsp90 co-chaperones [19].

Our data are in agreement with the results obtained by Takahashi et al. that showed that the plant CHORD containing protein, rar1, interacts with Hsp90. Moreover, rar1 also

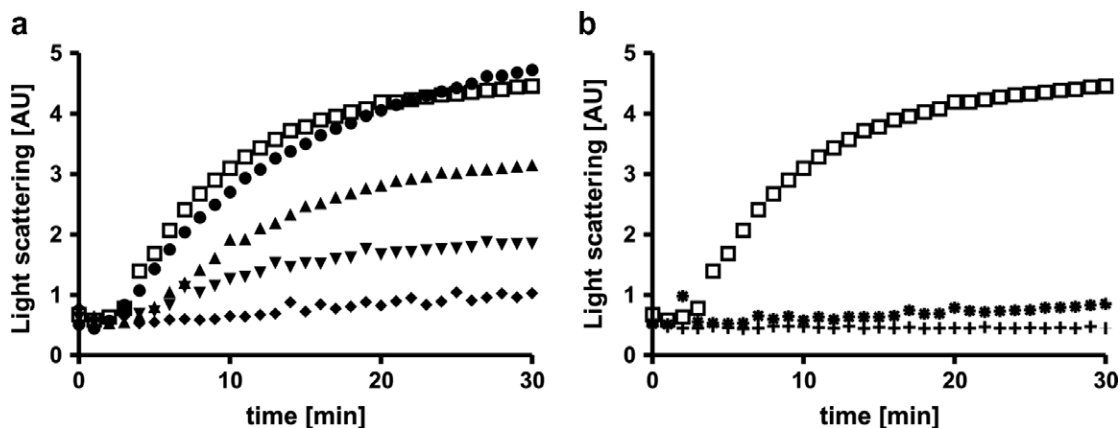


Fig. 5. Melusin suppresses the heat induced aggregation of citrate synthase. (a) Melusin activity was tested in citrate synthase aggregation assay. Aggregation of citrate synthase (200 nM) at 45 °C caused light scattering (□). 200 nM GST alone (●) did not prevent the citrate synthase aggregation, whereas GST–melusin at 50 nM (▲), 100 nM (▼), 200 nM (◆) suppressed the thermal induced aggregation effect in a dose dependent manner. (b) Control experiments showed that 200 nM GST (\*) and 200 nM GST–melusin (+) did not undergo heat induced aggregation, as observed for 200 nM Citrate synthase (□).

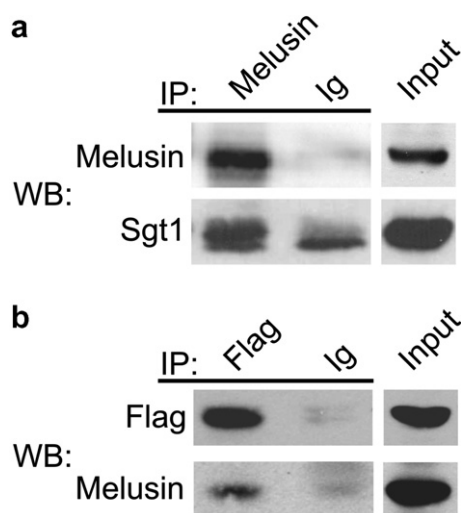


Fig. 6. Melusin binds to Sgt1. (a) Melusin was immunoprecipitated (IP) from COS cells expressing Myc-melusin using an anti-melusin antibody and an unrelated antibody as control (Ig). Western blot (WB) analysis with anti-Sgt1 and melusin antibodies were performed. (b) Protein extract from COS cells expressing Myc-melusin and flag-Sgt1 were immunoprecipitated with an anti-flag antibody to precipitate Sgt1 and with an unrelated antibody as control (Ig). The immunoprecipitated proteins were revealed by western blot analysis with anti-melusin and anti-flag antibodies.

binds to Sgt1, a molecule that acts, together with Hsp90 and rar1, in disease resistance in plants [20]. By co-immunoprecipitation assays we show that melusin is also capable to associate with Sgt1 in mammalian cells, confirming that the CHORD-containing protein molecular machinery is evolutionary conserved.

In summary here we demonstrate that melusin is a new Hsp90 interactor and has a chaperone activity per se. On the basis of these findings we suggest that melusin ability to affect signal transduction in heart hypertrophy can be exerted both by its own chaperone activity on crucial signaling components or by driving Hsp90 on particular transduction pathways.

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## References

- [1] Brancaccio, M., Guazzzone, S., Menini, N., Sibona, E., Hirsch, E., De Andrea, M., Rocchi, M., Altruda, F., Tarone, G. and Silengo, L. (1999) Melusin is a new muscle-specific interactor for beta(1) integrin cytoplasmic domain. *J. Biol. Chem.* 274, 29282–29288.
- [2] Shirasu, K., Lahaye, T., Tan, M.W., Zhou, F., Azevedo, C. and Schulze-Lefert, P. (1999) A novel class of eukaryotic zinc-binding

proteins is required for disease resistance signaling in barley and development in *C. elegans*. *Cell* 99, 355–366.

- [3] Brancaccio, M., Menini, N., Bongioanni, D., Ferretti, R., De Acetis, M., Silengo, L. and Tarone, G. (2003) Chp-1 and melusin, two CHORD containing proteins in vertebrates. *FEBS Lett.* 551, 47–52.
- [4] De Acetis, M., Notte, A., Accornero, F., Selvetella, G., Brancaccio, M., Vecchione, C., Sbroggiò, M., Collino, F., Pacchioni, B., Lanfranchi, G., Aretini, A., Ferretti, R., Maffei, A., Altruda, F., Silengo, L., Tarone, G. and Lembo, G. (2005) Cardiac overexpression of melusin protects from dilated cardiomyopathy due to long-standing pressure overload. *Circ Res* 96, 1087–1094.
- [5] Brancaccio, M., Fratta, L., Notte, A., Hirsch, E., Poulet, R., Guazzzone, S., De Acetis, M., Vecchione, C., Marino, G., Altruda, F., Silengo, L., Tarone, G. and Lembo, G. (2003) Melusin, a muscle-specific integrin beta1-interacting protein, is required to prevent cardiac failure in response to chronic pressure overload. *Nat Med* 9, 68–75.
- [6] Fontana, J., Fulton, D., Chen, Y., Fairchild, T.A., McCabe, T.J., Fujita, N., Tsuruo, T. and Sessa, W.C. (2002) Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ. Res.* 90, 866–873.
- [7] Walerych, D., Kudla, G., Gutkowska, M., Wawrzynow, B., Muller, L., King, F.W., Helwak, A., Boros, J., Zylicz, A. and Zylicz, M. (2004) Hsp90 chaperones wild-type p53 tumor suppressor protein. *J. Biol. Chem.* 279, 48836–48845.
- [8] Tolson, J.K. and Roberts, S.M. (2005) Manipulating heat shock protein expression in laboratory animals. *Methods* 35, 149–157.
- [9] Wawrzynow, B., Zylicz, A., Wallace, M., Hupp, T. and Zylicz, M. (2007) MDM2 chaperones the p53 tumor suppressor. *J. Biol. Chem.* 282, 32603–32612.
- [10] Buchner, J., Grallert, H. and Jakob, U. (1998) Analysis of chaperone function using citrate synthase as nonnative substrate protein. *Methods Enzymol.* 290, 323–338.
- [11] Pellegrino, M., Provero, P., Silengo, L. and Di Cunto, F. (2004) CLOE: identification of putative functional relationships among genes by comparison of expression profiles between two species. *BMC Bioinform.* 5, 179.
- [12] Brown, M.A., Zhu, L., Schmidt, C. and Tucker, P.W. (2007) Hsp90 – from signal transduction to cell transformation. *Biochem. Biophys. Res. Commun.* 363, 241–246.
- [13] Zou, J., Guo, Y., Guettouche, T., Smith, D.F. and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94, 471–480.
- [14] Pratt, W.B. and Toft, D.O. (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp. Biol. Med.* (Maywood) 228, 111–133.
- [15] Takahashi, A., Casais, C., Ichimura, K. and Shirasu, K. (2003) HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 100, 11777–11782.
- [16] Hahn, J.S. (2005) Regulation of Nod1 by Hsp90 chaperone complex. *FEBS Lett.* 579, 4513–4519.
- [17] Pearl, L.H. (2005) Hsp90 and Cdc37 – a chaperone cancer conspiracy. *Curr. Opin. Genet. Dev.* 15, 55–61.
- [18] Catlett, M.G. and Kaplan, K.B. (2006) Sgt1p is a unique co-chaperone that acts as a client adaptor to link Hsp90 to Skp1p. *J. Biol. Chem.* 281, 33739–33748.
- [19] Garcia-Ranea, J.A., Mirey, G., Camonis, J. and Valencia, A. (2002) p23 and HSP20/alpha-crystallin proteins define a conserved sequence domain present in other eukaryotic protein families. *FEBS Lett.* 529, 162–167.
- [20] Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K. and Schulze-Lefert, P. (2002) The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. *Science* 295, 2073–2076.